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Enhancement of intranasal vaccination in mice with deglycosylated chain A ricin by LTR72, a novel mucosal adjuvant

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Abstract

Intranasal (i.n.) vaccination with two suboptimal doses of 8 μg of deglycosylated chain A ricin (DGCA) stimulated low anti-ricin ELISA IgG and neutralizing antibody responses and the vaccine was only marginally protective against a lethal ricin toxin aerosol challenge. However, in the presence of 4, 2, or 1 μg of the mucosal adjuvant LTR72, a mutant of the heat-labile enterotoxin of *Escherichia coli*, the low antibody response and protection were substantially enhanced. In comparison to the vaccination with DGCA alone, vaccination with DGCA in the presence of three dose levels of LTR72, the anti-ricin ELISA serum IgG geometric mean titer (GMT) was increased, respectively, 191-, 572-, and 51-fold for IgG; 91-, 93-, and 60-fold for IgG1; nine-, six-, and two-fold for IgG2a; zero-, two-, and zero-fold for IgA. The three dose levels of the adjuvant enhanced the anti-ricin ELISA immunoglobulin GMTs in the lung lavage 4-, 14-, and 7-fold for IgG; two-, five-, and six-fold for IgG1; two-, six-, and two-fold IgG2a; and zero-, three-, and zero-fold for IgA, respectively. Compared to GMT obtained with the aqueous vaccine (1:2), the 10% serum neutralizing antibody GMT for the three dose levels was enhanced 25-, 60-, and 62-fold, respectively while the 50% neutralizing antibody GMT was enhanced more than 3-, 19- and 10-fold. Only 20% of the mice immunized with DGCA survived the lethal whole body aerosol challenge with 5–10 LD_{50} ricin toxin, while in the presence of 4, 2, and 1 μg LTR72, 100, 100 and 90% of the vaccinated mice survived, respectively.

Safety of administration of two doses of LTR72 is indicated by the absence of histopathological changes in every organ including the lung and the CNS of the mice during the vaccination and during 57 days of the study. In the nasal passages of the mice in the absence of DGCA, LTR72 caused a transient inflammation for less than 7 weeks without permanent epithelial changes. Administration of the adjuvant in the presence of DGCA did not cause additional changes.

Compared to the surviving mice vaccinated with DGCA alone, administration of the mucosal adjuvant with DGCA in spite of the better efficacy did not attenuate the lung injury at a single time point (16 days post-challenge). In mice treated with high(er) dose of vaccine, histological examinations during longer observation period rather than at one time point could reveal a different pattern.
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Keywords: Intranasal vaccination; Deglycosylated chain A ricin; Mucosal adjuvant

1. Introduction

Mucosal surfaces of the nasal passages are the major portals of entry for air-borne microorganisms and bioterrorism agents, including ricin toxin, therefore, the mucosal surfaces constitute the first line of defense. Because most parenterally administered "classical" vaccines by themselves are only partially effective in inducing mucosal immunity, and many recombinant vaccine candidates are poorly antigenic, there is a substantial interest in developing and applying adjuvants that can enhance mucosal immunity.

Parenteral administration of aluminum (alum) hydroxide or phosphate (the approved adjuvants for human use) with ricin toxoid completely protected mice from a low to moderate aerosol-delivered ricin challenge (J. Hewetson, personal communication). However, alum adjuvant given by intranasally (i.n.) or aerosol route may cause adverse

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14. ABSTRACT

Intranasal (i.n.) vaccination with two suboptimal doses of 8mug of deglycosylated chain A ricin (DGCA) stimulated low anti-ricin ELISA IgG and neutralizing antibody responses and the vaccine was only marginally protective against a lethal ricin toxin aerosol challenge. However, in the presence of 4, 2, or 1mug of the mucosal adjuvant LTR72, a mutant of the heat-labile enterotoxin of Escherichia coli, the low antibody response and protection were substantially enhanced. In comparison to the vaccination with DGCA alone, vaccination with DGCA in the presence of three dose levels of LTR72, the anti-ricin ELISA serum IgG geometric mean titer (GMT) was increased, respectively, 191-, 572-, and 51-fold for IgG; 91-, 93-, and 60-fold for IgG1; nine-, six-, and two-fold for IgG2a; zero-, two-, and zero-fold for IgA. The three dose levels of the adjuvant enhanced the anti-ricin ELISA immunoglobulin GMTs in the lung lavage 4-, 14-, and 7-fold for IgG; two-, five-, and six-fold for IgG1; two-, six-, and two-fold IgG2a; and zero-, three-, and zero-fold for IgA, respectively. Compared to GMT obtained with the aqueous vaccine (1:2), the 10% serum neutralizing antibody GMT for the three dose levels was enhanced 25-, 60-, and 62-fold, respectively while the 50% neutralizing antibody GMT was enhanced more than 3-, 19- and 10-fold. Only 20% of the mice immunized with DGCA survived the lethal whole body aerosol challenge with 5-10 LD(50) ricin toxin, while in the presence of 4, 2, and 1mug LTR72, 100, 100 and 90% of the vaccinated mice survived, respectively. Safety of administration of two doses of LTR72 is indicated by the absence of histopathological changes in every organ including the lung and the CNS of the mice during the vaccination and during 57 days of the study. In the nasal passages of the mice in the absence of DGCA, LTR72 caused a transient inflammation for less than 7 weeks without permanent epithelial changes. Administration of the adjuvant in the presence of DGCA did not cause additional changes. Compared to the surviving mice vaccinated with DGCA alone, administration of the mucosal adjuvant with DGCA in spite of the better efficacy did not attenuate the lung injury at a single time point (16 days post-challenge). In mice treated with high(er) dose of vaccine, histological examinations during longer observation period rather than at one time point could reveal a different pattern.

15. SUBJECT TERMS

ricin, vaccine, A-chain, recombinant, deglycosylated, laboratory animals, mice

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reactions and most likely will not be approved for administration by these routes. Ricin vaccine administered i.n. or by aerosol in the presence of a known mucosal adjuvant might stimulate full protection without the occurrence of lung injury due to effective stimulation of sIgA antibodies in the lower respiratory tract. The extent and the length of lung injury was characterized in mice that were vaccinated by three parenteral doses of DGCA (G.M. Zaucha, personal communication). All vaccinated mice survived the lethal ricin aerosol challenge; however, by Day 14 they developed significant pulmonary changes. By Day 28 post-challenge, the histopathological changes were reduced in incidence and severity, but the changes were statistically significant compared to non-challenged controls. By Day 56, the lung damage was generally minimal, though still statistically significant. The only finding remained at Day 112 was a statistically significant, minimal to mild lymphoplasmocytic perivasculitis, affecting all mice.

The heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli strains* [1] acts as a potent mucosal adjuvant and induces an immune response against the antigen coadministered by i.n. or parenteral route, where most adjuvants are unable to induce an immune response [2]. However, LT's toxicity precludes its use in humans. Like cholera toxin from *Vibrio cholerae* strains, LT toxin is composed of two functionally distinct domains: the enzymatically active 27 kD A subunit [3,4] with ADP-ribosyltransferase activity [5–7], and the pentameric 60 kD [4] B subunit [8,9] that contains the monosialoganglioside (GM-1) receptor-binding site that binds to the receptor on the surface of epithelial cells, leading to LT internalization. The A subunit intoxicates eukaryotic cells by activating G-proteins GTP-binding proteins that regulate the levels of the secondary messenger cAMP [10,11]. Increased levels of cAMP alters ion transport, inducing secretion of water and chloride ions in the intestine [12].

Guided by the crystal structure of LT and by molecular modeling, site-directed mutagenesis was used to replace a single amino acid within the enzymatic A subunit of LT, resulting in two mutants, LTR72 (Ala → Arg) and LTK63 (Ser → Lys), with <1%, or no enzymatic activity of the native LT, respectively [13]. Both of these mutants have been shown to be potent mucosal adjuvants, after administration by a number of routes [14]. At a dose of 1 μg, LTR72 exhibited a mucosal adjuvanticity, similar to that observed with wild-type LT. This trend was consistent for both the amounts and the kinetics of ovalbumin-specific antibody induced, and priming of antigen-specific T lymphocytes [14]. Combined i.n. vaccination of mice with influenza vaccine and LTR72 significantly enhanced the antibody response compared with the response elicited by vaccine given by the traditional parenteral route [15]. Intranasal vaccination also induced mucosal IgA antibody responses, while systemic vaccination did not. Furthermore, i.n. vaccination with influenza vaccine and LTR72 induced a high IgG2a response in the serum [15]. Intranasal vaccination with ovalbumin in the presence of LTR72 increased to about 30- and 50-fold the IgA levels in the nasal washings and in the serum of mice, respectively, comparable to IgA responses stimulated i.n. by ovalbumin and with wild-type LT. Intranasal vaccination by the alternative adjuvant LTK63 induced 170- and 190-times higher serum IgG and nasal IgA responses, respectively, compared to responses elicited by influenza vaccine alone [16].

In our study, we evaluated LTR72 as a mucosal adjuvant combined with DGCA vaccine administered i.n. Stimulation of the anti-ricin systemic and mucosal immune response and the protection against aerosol-delivered lethal ricin challenge were evaluated. Histological samples were prepared 5–6 days after administering each of the two ineffective vaccine doses and after the challenge, to rule out vaccine- and/or adjuvant-related toxicity in all of the organs. The possible attenuation of the ricin-related lung injury in the surviving mice vaccinated in the presence of the mucosal adjuvant was assessed by histopathology 16 days post-challenge.

2. Materials and methods

2.1. Vaccine

DGCA ricin was produced by Perimmune In., Gaithersburg, MD, USA according to Good Manufacturing Practices regulations. The vials contained 100 μl of lyophilized vaccine, which was reconstituted with sterile saline 1 day before use. The reconstituted vaccine dose with or without adjuvant was prepared in a volume of 24, and 12 μl was instilled into each nostril.

2.2. Mucosal adjuvant

LTR72 was made by Chiron Co., Siena, Italy and was provided to our laboratory by a Material Transfer Agreement. The adjuvant was stored in a buffer solution at 4 °C. Before use, the desired dose was mixed with the vaccine.

2.3. Mice

Female BALB/c 8–10-week-old mice were obtained from the National Cancer Institute, Frederick Research and Development Facility at Frederick, MD, USA. The mice were housed in full compliance with the Guide for Care and Use of Laboratory Animals. The mice were fed food and water ad libitum. The vaccine and/or the adjuvant were administered without anesthesia.

For histology, the mice were euthanized with 0.1 ml intraperitoneal injection of euthasol (390 mg of pentobarbital sodium/1.0 ml). The organs were removed and were placed in a 10% formaldehyde solution and stored until they were examined. To collect lung lavage samples, the mice were euthanized by euthasol, and a volume of 5.0 ml of sterile phosphate-buffered saline (PBS) was infused through the trachea into the lungs. The collected fluid was stored at −20 °C until used for ELISA and neutralization tests.

2.4. Vaccination

Mice were vaccinated i.n. on Days 0 and 28 with 8 μg DGCA with or without LTR72 for survival studies, for histology and for lung lavage. For histology five mice were euthanized on Days 6, 34, or 57. For lung lavage, on Day 40, five mice/group were euthanized and the bronchoalveolar lung (BAL) lavage was collected with 5.0 ml of PBS. For the survival study, the mice (10/group) were bled from the periorbital venous sinus on Day 40. Before being bled, the mice were anesthetized with a mixture of ketamine–acepromazin–xylazine, which was administered in the caudal thigh muscle. The serum samples were stored at −20 °C until their ELISA and their neutralizing anti-ricin antibody titers were determined. On Day 47, the mice were challenged with five LD_{50} of ricin toxin (Vector Laboratories Burlingame, CA, USA).

For the survival study, mice were exposed to ricin in a dynamic, whole-body exposure chamber with a total system airflow rate of rate of 19.51 min^{-1}. A small-particle aerosol with a mass median aerodynamic diameter of 1.2 μm was generated by a collision nebulizer. The aerosol was sampled during the entire exposure period of 10 min by using a standard all-glass impinger containing 10 ml of sterile PBS, pH 7.4. Ricin toxin in the collection fluid was analyzed for protein (Pierce Micro BCA, Pierce Labs, Rockford, IL, USA). Respiratory minute volumes were estimated by using Guyton's formula, which is based on animal weight [17]. The estimated inhaled dose of 60 μg/kg was calculated from the respiratory breathing rates and the aerosol concentration of the toxin. The exposed mice were observed daily for 3 weeks.

To determine ricin-related lung injury among the challenged, surviving mice, four or five were euthanized for histology 16 days post-challenge, which was beyond the ricin-related death.

2.5. In vitro ricin neutralization assay

In vitro neutralization of the protein synthesis of EL-4 (Ehrlich ascites) cells was used to measure neutralizing antibodies to ricin as reported [18]. Cultured EL-4 cells (ATCC-TIB39) were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum. Ricin was pre-titrated for cytotoxicity in the cell assay. Cells were adjusted to 5×10^5 ml^{-1} in leucine-free medium and 100 μl was added to wells of a sterile 96-well Costar (#25860) microplate. Fifty microlitre of ricin at concentrations ranging from 2 to 50 ng was added to the wells and the cells were incubated overnight at 37 °C with 5% CO_2 pressure. Fifty microlitre of [^{3}H]leucine (Amersham; specific activity 143 Ci/mmol) was added and the cells were incubated 4 h at 37 °C under 5% CO_2. The cells were then harvested with a Skatron cell harvester onto cellulose mats and the mats were counted in a BetaPlate 1205 liquid scintillation counter. A ricin cytotoxicity curve was then plotted and the ricin dose that killed 100% of the cells was determined. The dose reproducibly was 20 ng/ml. For the neutralization assay, serial half-log dilutions of the serum were mixed in equal volumes with the ricin at 40 ng/ml (final ricin concentration: 20 ng/ml) and added to the cell plates as described above. A ricin standard curve was always included with each assay. The neutralization titer was defined as the highest dilution of the serum that protected at least 10% of the cells from ricin-induced cytotoxicity. *Tpv*-value when 10% of the cells are protected was 1×0^{-8}, which is +8 standard deviation.

2.6. Determination of anti-ricin IgG iso- and subisotypes by ELISA

Blood samples and lung lavage fluids were collected from the mice at the times indicated. The levels of anti-ricin IgG, IgG1, IgG2a, and IgA in the serum and in the lung lavage of each mouse were measured by direct ELISA using 0.25 μg/well of ricin as the capture antigen. Into three-fold serial dilutions of 50 μl of the test samples 50 μl of peroxidase-labeled anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg MD, USA) or IgA or IgG2a or IgG1 (ICN Biomedicals Inc., Irvine, CA, USA) was added. After adding 50 μl of *o*-phenylenediamine (OPD) substrate (Sigma Chemical Co., St. Louis, MO, USA), we incubated the plates for 20 min at room temperature. Stop buffer (10 μl) (0.1 N NaOH) was added, and the optical density (OD) of each well was read at 405 nm with ELISA plate reader (Model MR 600, Dynatech Co., Chantilly, VA, USA). Antibody levels were expressed as the reciprocal of the GMT based on the last serum dilution with an OD twice that of the control (normal) serum at the same dilution. In that case, the *p*-value was 0.01, provided that the OD of the normal serum was ∼0.1–0.2. In titrations of immune and normal serum, the initial serum dilution was 1:100, and if that dilution was negative, the serum's base-line titer was taken as 1:10. The *p*-values of the GMTs were calculated by Wilcoxon non-parametric and *t* estimation procedures with SAS-assisted software.

3. Results

3.1. Determination of the suboptimal vaccination dose without LTR72

To find the suboptimal vaccination dose which would protect 10–40% of the mice, the animals were vaccinated by two i.n. doses of 40, 20, 10, 5, or 2.5 μg of DGCA and were challenged by the schedule and procedure indicated in Section 2. The GMT iso- and subisotype ELISA anti-ricin antibody titers are shown in Fig. 1. Only those mice that received two doses of 40 μg were protected against lethal aerosol-delivered ricin challenge, and these mice had the highest IgG iso- and subisotype and 1:164 neutralizing antibody titers. The neutralizing antibody titer(s) are not shown in the figure, as only 40 μg of DGCA stimulated neutralizing antibody titers, while

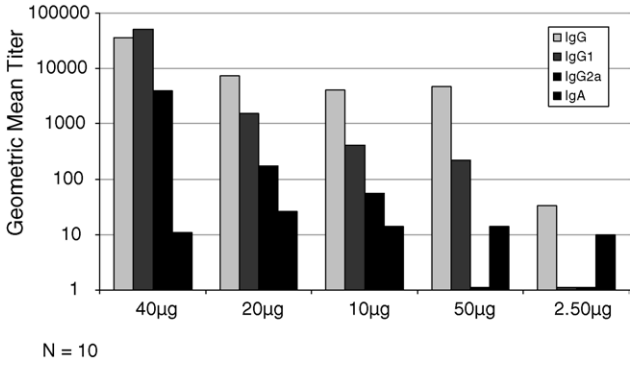

Fig. 1. Geometric mean anti-ricin serum ELISA IgG, IgG1, IgG2a and IgA titers of mice ($N = 10$) vaccinated i.n. with various doses of DGCA ricin vaccine on Days 0 and 28 and were bled on Day 40.

lower doses did not. The dose of 40 μg protected all 10 mice in this group (not shown in the figure), while 20 μg protected slightly more than half the vaccinated mice (60%). The mice in the later vaccination group had 10 times lower IgG iso- and subisotypes GMTs compared to results from those mice vaccinated with the highest dose. The doses of 10 and 5 μg stimulated marginal (20%) protection and the IgG1 and IgG2a GMTs declined further; however, the IgG titers remained relatively high. The dose of 2.5 μg failed to protect and the ELISA iso- and subisotype GMTs were absent or were very low. The GMT of the anti-ricin IgA response in the serum was low. The majority of the mice had undetectable levels at the 1:10 initial serum dilution. Based on the dose titration study, by probit analysis 8 μg was the calculated dose which would protect 20% of the mice. Therefore, to evaluate the adjuvant's efficacy, the DGCA dose was 8 μg.

3.2. Vaccination of mice by DGCA with/without LTR72

3.2.1. Enhancement of the serum ELISA IgG iso- and subisotypes

As described in Section 2, groups of mice were vaccinated i.n. with a suboptimal dose of 8 μg of DGCA in the presence of 4, 2, or 1 μg of LTR72. Before challenge, the mice (10 mice/group) were bled to determine their ricin-specific ELISA and neutralizing antibody response. Additional mice (five mice/group) were euthanized to collect lung lavage for ELISA and neutralizing antibody determinations.

Compared to the serum IgG ELISA GMT titer of DGCA alone (1:142), the anti-ricin IgG GMT titers in the presence of 4 (1:27,222), 2 (1:81,365), and 1 μg (1:7257) were enhanced 191-, 572-, and 51-fold, respectively (Fig. 2). The IgG1 serum ELISA GMTs were enhanced by LTR72 up to 572- and 93-fold, respectively (Fig. 2). The respective IgG1 GMT's were enhanced by 91-, 93- and 60-fold (Fig. 2). The enhancement of the IgG and IgG1 ELISA titers by all three doses of the adjuvant were highly significant both by Wilcoxon and *t* approximation methods of *p*-value calculations (Table 1). Compared with the IgG2a GMT stimulated with the vaccine alone (1:33), the GMT was enhanced nine-, seven-, and two-

Table 1
p-values* of immunoglobulin iso- and subisotype or neutralization (NT) titers of mice

	Serum						Lung lavage					
	DGCA + 4 μg LTR72		DGCA + 2 μg LTR72		DGCA + 1 μg LTR72		DGCA + 4 μg LTR72		DGCA + 2 μg LTR72		DGCA + 1 μg LTR72	
	Wilcoxon	*t* approx.	Wilcoxon	*t* approx.	Wilcoxon	*t* approx.	Wilcoxon	*t* approx.	Wilcoxon	*t* approx.	Wilcoxon	*t* approx.
IgG	0.0002	0.0016	0.0001	0.0012	0.0012	0.0043	0.0371	0.0706	0.0219	0.0278	0.0181	0.0424
IgG1	0.0004	0.0023	0.0003	0.0018	0.0009	0.0036	0.0746	0.1125	0.0088	0.0018	0.0099	0.0298
IgG2a	0.0001	0.0007	0.0022	0.0064	0.0776	0.0937	0.1312	0.1696	0.0720	0.1055	0.4237	0.4443
IgA	1.0000	1.0000	0.0776	0.0937	1.0000	1.0000	0.5186	0.5347	0.1887	0.2212	0.6985	0.7075
10%NT	0.0009	0.0039	0.0002	0.0016	0.0006	0.0034	–	–	–	–	–	–
50%NT	0.0776	0.0937	0.0060	0.0128	0.0350	0.0485	–	–	–	–	–	–

The *p*-values of the serum and lung lavage immunoglobulin ELISA iso- and subisotype (shown in Figs. 2 and 3), and the 10 and 50% neutralizing (NT) geometric mean titers in the serum of mice (shown in Fig. 4).
* Compared to the titers of DGCA vaccination.

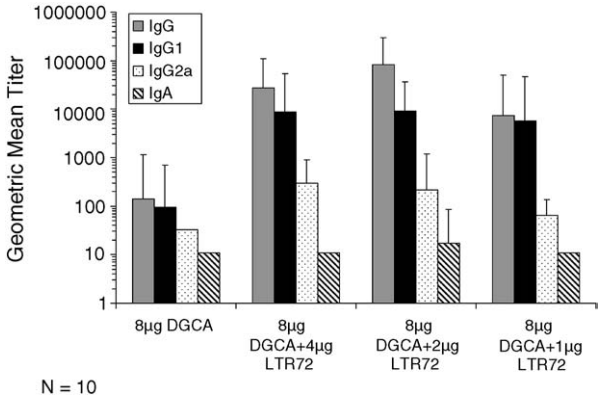

Fig. 2. Geometric mean anti-ricin serum ELISA IgG, IgG1, IgG2a and IgA titers of mice ($N = 10$) vaccinated i.n. with a suboptimal dose (8 μg) of DGCA with or without LTR72 on Days 0 and 28 and were bled on Day 40.

fold with 4, 2, or 1 μg of LTR72, respectively (Fig. 2). The *p*-values of IgG2a enhancement were highly significant with 4 and 2 μg, but not with 1 μg (Table 1). The GMT of IgA in the serum was not enhanced significantly by the mucosal adjuvant (Fig. 2).

3.2.2. Enhancement of the ELISA iso- and subisotypes in the BAL

Compared to GMT (1:3) in the serum of mice vaccinated with DGCA alone, the respective anti-ricin IgG GMT of 1:12, 1:42 and 1:22 was enhanced 4-, 14-, and 7-fold by 4, 2, or 1 μg dose levels of the adjuvant (Fig. 3). All the *p*-values (with the exception of *t* approximation of the highest dose) were significant when compared to the GMT of DGCA alone (Table 1). The increase in the GMTs of IgG1 was more modest with the three doses levels of the adjuvant, two-, five-, and six-fold (Fig. 3), respectively. The *p*-values were significant with 2 and 1 μg of LTR72, but not with 4 μg (Table 1). Three dose levels of the adjuvant enhanced the IgG2a GMT only moderately, while IgA titers were changed less (Fig. 3),

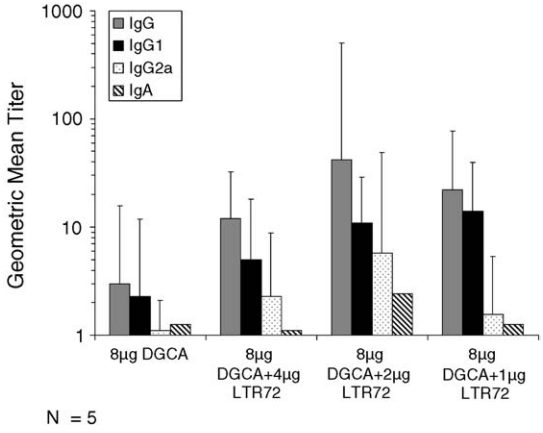

Fig. 3. Geometric mean anti-ricin lung lavage ELISA IgG, IgG1, IgG2a, and IgA titers of mice ($N = 5$) vaccinated i.n. with a suboptimal dose (8 μg) of DGCA with or without LTR72 ricin vaccine on Days 0 and 28. The mice were euthanized, bled and the BAL was collected on Day 40.

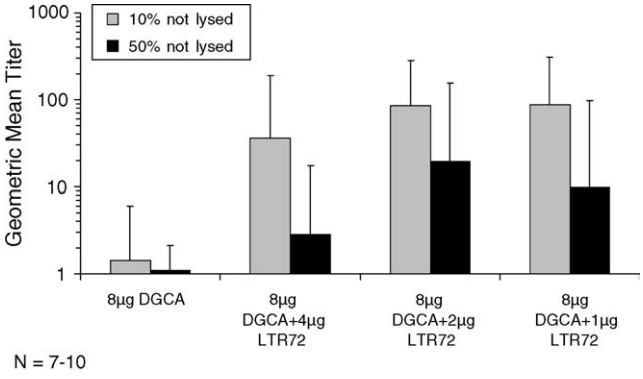

Fig. 4. Geometric mean anti-ricin serum 10 and 50% neutralizing antibody titers of mice ($N = 7$–10) vaccinated i.n. with a suboptimal dose (8 μg) of DGCA with or without LTR72 on Days 0 and 28 and were bled on Day 40.

but *p*-values of IgG2a and the IgA titers increases were not significant (Table 1).

3.2.3. Enhancement of the neutralizing antibodies in the serum

The 10% ricin neutralization serum antibody GMT is shown in Fig. 4. Only one of 10 mice vaccinated with DGCA alone had a neutralizing antibody titer with a starting dilution of 1:10, consequently yielding a GMT of 1:2. All mice vaccinated by DGCA vaccine in the presence of 4, 2, and 1 μg of LTR72 had 10% neutralization antibody titers with the respective GMTs of 1:36, 1:86, and 1:88. The respective increases of 25-, 60-, and 62-folds were highly significant, as shown in Table 1. The 50% GMTs stimulated with 4, 2, and 1 μg of adjuvant were increased up to 20-fold.

3.2.4. Neutralizing antibodies in the BAL

Only one of five mice vaccinated with DGCA/2 μg of adjuvant and one of five mice vaccinated with DGCA/1 μg of adjuvant had 10% neutralizing antibody titers in the BAL (not shown in the figure). None of the five mice vaccinated with DGCA or with DGCA and 4 μg of adjuvant had measurable neutralizing antibodies in their undiluted BAL.

3.2.5. Resistance of vaccinated mice to lethal aerosol challenge

The mice were challenged with lethal ricin toxin 19 days after the second vaccination. All the unvaccinated control mice [10] died by Day 5, and only two of 10 mice survived when they were vaccinated with DGCA without the adjuvant (Fig. 5). However, 10 of 10 mice survived the lethal challenge when they were vaccinated with DGCA and 4 or 2 μg of LTR72, and nine of 10 mice survived that received the immunogen in conjunction with 1 μg of adjuvant.

3.3. Histology

Histopathological evaluation of all organs is summarized in Tables 2 and 3. Organs that were normal are not mentioned in the tables with the exception of the nasal passages, lungs

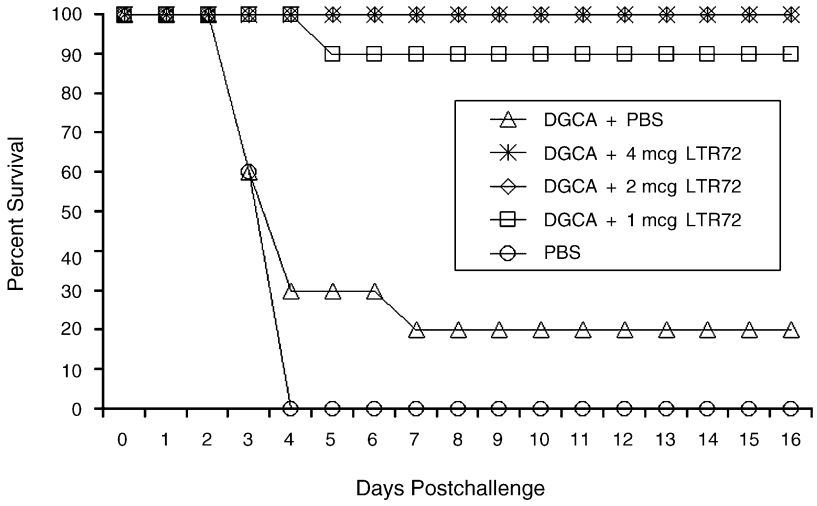

Fig. 5. Resistance of mice ($N = 10$) to aerosol ricin challenge on Day 47 elicited by i.n. vaccination with suboptimal dose (8 μg) of DGCA with or without LTR72 on Days 0 and 28.

and central nervous system (CNS) as they are the target organs of the mucosal adjuvant under evaluation and/or of the ricin toxin.

3.3.1. *Safety of i.n. administration in unchallenged mice*

Histopathology showed that 6 days after administration of the first i.n. administration of DGCA, minimal to mild multifocal olfactory and respiratory degeneration and necrosis accompanied by acute neutrophilic inflammation was observed in the mice (Table 2). In mice that received LTR72, the inflammation was more extenuated, but the inflammation was transient, since 6 days after the second dose (at Day 34) the nasal inflammation was observed only in a few animals as a result of administration of the three dose levels of the adjuvant. By Day 57, the nasal inflammation was minimal without epithelial changes, and was indistinguishable in the animals that received DGCA or the adjuvant alone, or the vaccine with the three dose levels of the adjuvant.

Lymphoid hyperplasia seen in the spleen, mesenteric, and mandibular lymph nodes 6 days after administration of DGCA or LTR72 represents an immune response to the nasal inflammation.

The lungs and the CNS were normal at every time point of the evaluation either with DGCA or with only the three doses of the adjuvant, or by their combined administration.

3.3.2. *Histopathology of mice vaccinated with/without adjuvant and challenged with ricin*

All non-vaccinated mice exposed to aerosol-delivered ricin died 5 days postexposure. Histopathology of moribund, non-vaccinated mice had ricin-related nasal inflammation, with epithelial degeneration, necrosis with scattered fibrinosuppurative exudates (Table 3). The lung lesions were characterized by moderate to severe bronchiolar and alveolar inflammation and by a moderate perivascular inflammation. Multifocal interstitial, perivascular, and alveolar edema

Table 2
Histology without ricin challenge

Immunization	Sacrificed	Histology
PBS	Day 6	Normal
DGCA + PBS; DGCA + 4 μg LTR72; 2 μg LTR72; 1 μg LTR72	Day 6	Minimal to mild (DGCA), moderate (LTR72) multifocal olfactory and respiratory degeneration and necrosis with acute neutrophilic inflammation; lungs and CNS: no lesions; lymphoid hyperplasia in mandibular, spleen and mesenteric lymph nodes.
PBS	Day 34	Normal
DGCA + PBS; DGCA + 4 μg LTR72; 2 μg LTR72; 1 μg LTR72	Day 34	Minimal to mild (DGCA), mild to moderate (in some LTR72) lymphocytic and neutrophilic inflammation; mild/moderate epithelial degeneration (in some LTR72) with fibrino-suppurative exudates; lungs and CNS normal.
PBS	Day 57	Normal
DGCA + PBS; DGCA + 4 μg LTR72; 2 μg LTR72; 1 μg LTR72	Day 57	Nasal lesions characterized by minimal multifocal lymphocytic inflammation, without epithelial changes; lungs and CNS normal.

Histology in the nasal passage, lungs, CNS and lymph nodes of mice vaccinated with suboptimal dose (8 μg) of DGCA with or without LTR72 on Days 0 and 28. Tissues were collected 6 days after the first and the second vaccinations, and at Day 57, which was 8 or 4 weeks after the first and second vaccinations, respectively.

Table 3
Histology 16 days after ricin challenge

	Nasal passages	Lungs
PBS	Mild multifocal lymphoplasmacytic and neutrophylic inflammation; scattered exudates; epithelial degeneration and necrosis.	Moderate to severe bronchiolar and alveolar fibrinonecrotic and suppurative inflammation; moderate perivascular inflammation and multifocal interstitial, perivascular and alveolar edema and hemorrhage.
DGCA + PBS	Mild multifocal lymphoplasmocytic and neutrophylic inflammation; no significant epithelial change.	Mild to moderate, multifocal peribronchiolar and perivascular lymphoplasmacytic and histiocytic inflammation; lymphoid hyperplasia of bronchiolar-associated lymphoid tissue (BALT).
DGCA + 4 μg LTR72	Multifocal mild lymphoplasmacytic and neutrophyilic subacute inflammation; no significant epithelial changes.	Moderate multifocal peribronchiolar and perivascular inflammation; lymphoid hyperplasia of BALT; also multifocal, mild alveolar, perivascular and interstitial edema.
DGCA + 2 μg LTR72	Mild multifocal lymphoplasmacytic and neutrophilic inflammation; no significant epithelial changes.	Mild to moderate, multifocal peribronchiolar and perivascular lymphoplasmacytic and histiocytic inflammation; lymphoid hyperplasia of BALT.
DGCA + 1 μg LTR72	Mild multifocal lymphoplasmacytic inflammation; no significant epithelial change.	Mild multifocal, peribronchiolar and perivascular lymphoplasmacytic and histiocytic inflammation accompanied by lymphoid hyperplasia of the BALT.

Histology in the nasal passage, lungs and bronchiolar-associated lymphoid tissue of mice 16 days after aerosol-delivered ricin toxin. The mice were vaccinated i.n. with a suboptimal dose (8 μg) of DGCA with or without LTR72 on Days 0 and 28, and the tissues were collected on Day 61.

and hemorrhage were well evident. In the nasal passages of the mice vaccinated with DGCA with/without LTR72, the inflammation was reduced to mild with no significant epithelial changes. Inflammation of the lung was also attenuated to mild to moderate without edema and hemorrhage in those mice that were vaccinated with DGCA with/without LTR72, with the exception of the mice that received DGCA and 4 μg of LTR72. These mice had a mild, alveolar, perivascular and interstitial edema. Lymphoid hyperplasia of bronchiolar-associated lymphoid tissue was seen in vaccinated mice with/without the adjuvant. No changes occurred in the CNS and no changes were seen in any other organs.

4. Discussion

We successfully used the mucosal adjuvant LTR72 to augment the efficacy DGCA vaccine administered twice by i.n. route. The aqueous vaccine stimulated only low neutralizing and ELISA antibody levels, consequently the mice were only marginally protected against lethal aerosol challenge. In contrast, all three doses of LTR72 considerably enhanced the anti-ricin humoral immune response and the resistance to lethal aerosol challenge.

We assessed the effect of three dose levels of the adjuvant on the ELISA antibody iso- and subisotype IgG as indicators of the Th-1 and Th-2 type immunity. The strongest enhancement among the isotypes was the serum IgG, but the serum IgG1 was enhanced nearly as strong, without a strong dose effect. The nearly three logs of enhancement of the suboptimal vaccination dose represents a very potent adjuvant effect.

The enhancement of IgG and IgG1 response by the adjuvant was also evident in the lungs as measured in the samples of the lung lavage. With the exception of the samples from the mice treated with the immunogen in the presence of the highest dose of LTR72, the levels of IgG and IgG1 were significantly higher than those from mice treated only with the vaccine. The presence of increased anti-ricin IgG in the lung undoubtedly augments the protection in the target organ of the ricin toxin. Low number of the samples (five) could be the reason that the vaccine in the presence of the highest dose of the adjuvant did not stimulate statistically significant higher IgG1 levels in the lungs.

Compared to IgG and IgG1, the enhancement of serum IgG2a was not as strong. The ratio of IgG1/IgG2a increased in the favor of IgG1, indicating that with the immunogen and the route of administration used in the study, the dominant immunity associated with the activity of the mucosal adjuvant was Th-2 type. These results conform with published data regarding the type of immune responses stimulated by LTR72 [19,20].

Relative to the DGCA, the 10% neutralization antibody response was enhanced with the mucosal adjuvant up to 62-fold. While DGCA alone did not stimulate the 50% neutralization titer, in the presence of LTR72, this titer was increased up to 20-fold. Most recent studies with LTK63 mucosal adjuvant combined with conjugated group C meningococcal vaccine yielded similar results in respect to enhancement of antibodies with bactericidal activity, which is known to correlate with the efficacy [21].

In our study, the number of lung lavage samples (five/group) was not sufficient to make a conclusion regarding the enhancement of the neutralization titer in the lungs by DGCA and LTR72. Only one out of five animals had an increase in the 10% neutralization titer in the presence of 2 or 1 μg, respectively, but none with the vaccine alone (results are not shown).

Very much the same case can be made with the sIgA titers in the lungs which were elevated by the presence of the adjuvant, but had no statistical significance. In the lung lavage process 5.0 ml of PBS was used, and such an amount could dilute out the neutralizing antibody titer of the sIgA content of the lungs, which is not very high. Recovering sIgA from the lungs with a smaller amount (1.0 ml) of wash could yield different results.

Furthermore, when higher dose of recombinant ricin vaccine was administered in the presence of the mucosal adjuvant LTK63, higher IgA was stimulated in the serum and in the lung (M. Kende, unpublished observation). LTK63 in conjunction with the recombinant ricin vaccine stimulated significantly higher neutralizing antibody titers not only in the serum, but also in the lung. In future preclinical studies and in, for a potential human use, a full dose of the vaccine will be used in conjunction with LTK63, and in that situation, the sIgA and IgA stimulation is expected to be more robust.

The high anti-ricin IgG and IgG1 ELISA and neutralizing antibody titers elicited by LTR72 are predictive for the resistance against lethal whole-body aerosol ricin challenge, as indicated by the complete enhancement of the protection. The 20% protection stimulated by a suboptimal dose of 8 μg of DGCA was augmented to 90–100% with 1–4 μg doses of the adjuvant. The single death in the group vaccinated with DGCA and 1 μg of LTR72 could have been caused by improper i.n. administration of the vaccine/adjuvant mixture. Or perhaps the mouse failed to inhale the delivered inoculums. Consequently, the lowest dose of 1 μg is probably as effective as the 4 μg, which agrees with the published results with LTR72 [13], which were obtained by three i.n. doses of the immunogen and the adjuvant in contrast to two doses administered in our study.

The wild-type LT adjuvant administered i.n. is known to reach the CNS via the olfactory nerve, therefore, it is excluded from human use. In contrast, as our study demonstrated, LTR72 did not affect the CNS, or any other organs during and after its administration for a period of 57 days of observation. Transient mild nasal inflammation was caused by the adjuvant, which was stronger only after the first dose than the inflammation caused by the vaccine. However, after the second dose, the inflammation due to the adjuvant was noticed in fewer animals and by Day 57, it was indistinguishable from the nasal inflammation due to the vaccine itself. Furthermore, the inflammation did not cause permanent epithelial changes, Consequently, the adjuvant could be safe for human use. LTR72, together with the other mutant LTK63, are now being developed for clinical testing in human volunteers. LTK63 has already been evaluated in a phase I clinical trial in about 100 human patients in Europe and Phase II clinical trial is planned in the spring of 2004. (G. DelGiudice, personal communications).

By administration of LTR72 with the vaccine, we wished to reduce or altogether to prevent lung-injury in vaccinated mice that survived ricin challenge. In comparison with the non-vaccinated, moribund mice, which were euthanized 12–24 h before the expected death, the inflammation was reduced to mild with no significant epithelial changes in the nasal passages of the treated mice with/without adjuvant. At 16 days post-challenge, although the lung injury was reduced by the vaccination, the presence of LTR72 did not further reduce it and did not prevent the inflammation more than the vaccine did. All the mice showed no signs of edema and hemorrhage as a result of vaccination with/without the adjuvant, with the exception of one mouse, which received the immunogen in the presence of 4 μg of LTR72, and which had a mild alveolar, perivascular and interstitial edema. The presence of lung edema in the mouse at 16 days cannot be explained, as mice surviving at 16 days survived indefinitely.

The inability of the adjuvant to reduce or prevent lung injury in conjunction with a suboptimal dose of the ricin vaccine is probably due to insufficient amount of IgA stimulated by the low antigenic mass. Histopathology of the lung injury at several time points for 60 days after challenge of the mice treated with high(er) dose of vaccine in conjunction with the mucosal adjuvant could present a different resolution as opposed to one time-point which was spaced at the height of the lung injury. Studies of that nature will be conducted in the near future using a recombinant mutant ricin immunogen without enzymatic activity. In all other aspects LTR72 exhibited excellent adjuvant activity, and it is expected to be very valuable in human use for i.n. or oral vaccination.

DGCA is without the B chain of the ricin, therefore, DGCA cannot bind to the galactosyl cell surface receptor. Therefore, triggering endocytic uptake is absent. DGCA can enter the epithelial cells lining of the nasal passages via the less efficient pinocytosis; however, when complexed with LTR72, DGCA probably enters the cells via the GM_{21} combining-sites on the B subunits of the adjuvant. The adjuvant also initiates a variety of biological mediators, and due to these dual roles, LTR72 and other related recombinant LT adjuvants are among the most potent mucosal adjuvants with a high prospect for human use. LTR72 could facilitate vaccination via the i.n. or aerosol route, which, is an alternative method of vaccine administration and it can lead to self-administration of the immunogen/adjuvant.

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References

[1] Spengler BD. The heat-labile enterotoxin of *E. coli*. Microbiol Rev 1992;56:622–47.
[2] Clements JD, Yancy RJ, Finkelstein RA. Properties of homogeneous heat-labile enterotoxin from *E. coli*. Infect Immun 1980;29:91–7.
[3] Jackson RJ, Fujihashi K, Xu Amano J, Kiyono H, Elson CO, McGhee JR. Optimizing oral vaccines: induction of systemic and mucosal B–cell and antibody responses to tetanus toxoid by use of cholera toxin as an adjuvant. Infect Immun 1993;61:4272–9.
[4] Sixma TK, Pronk SE, Kalk KH, Wartna ES, van Zenten BEM, et al. Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli*. Nature 1991;351:371–7.
[5] Holmgren J. Actions of cholera toxin and the prevention and treatment of cholera. Nature 1981;292:413–7.
[6] Gill DM, Woolkalis MJ. Cholera toxin-catalyzed [^{32}P]ADP-ribosylation of proteins. Methods Enzymol 1991;195:267–80.
[7] Moss J, Vaughan M. ADP-ribosylation of guanyl nucleotide-binding regulatory proteins by bacterial toxins. Adv Enzymol Relat Areas Mol Biol 1988;61:303–79.
[8] Suguii ST. Binding specificities of heat labile enterotoxins isolated from porcine nd human enterotoxigenic *Escherichia coli* for different gangliosides. Can J Microbiol 1989;35:670–3.
[9] Holmgren J, Lonroth I, Svennerholm L. Tissue receptor for cholera exotoxin postulated structure from studies with GM-1 gangliosyde and related glycolipids. Infect Immun 1973;8:208–14.
[10] Rappuolli R, Pizza M. Structure and evolutionary aspects of ADP-ribosylating toxins. In: Alouf J, Freer J, editors. Sourcebook of bacterial protein toxins. Academic Press; 1991. p. 1–20.
[11] Moss J, Vaughan M. Toxin ADP-ribosyltransferases that act on adenylat-cyclase systems. Methods Enzymol 1984;106:411–8.
[12] Field M, Rao MC, Chang EB. Intestinal electrolyte transport and diarrheal disease. N Engl J Med 1989;321:800–6.
[13] Giuliani MM, Del Giudice G, Giannelli V, Dougan G, Douce G, et al. Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of *Escherichia coli* heat-labile enterotoxin with partial knockout of ADP-rybosyltransferase activity. J Exp Med 1998;187:1123–32.
[14] Rappuoli R, Pizza M, Douce G, Dougan G. Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins. Immunol Today 1999;20:493–500.
[15] Brackman JD, Ott G, O’Hagan DT. Intranasal immunization in mice with influenza vaccine in combination with the adjuvant LT-R72 induces potent mucosal and serum immunity which is stronger than that with traditional intramuscular immunization. Infect Immun 1999;67:4276–9.
[16] O’Hagen DT, Rappuoli R. Novel delivery systems for intranasal immunization with inactivated influenza vaccine. Drug Deliv Syst Sci 2001;1:41–6.
[17] Guyton AC. Measurement of respiratory volumes of laboratory animals. Am J Phys 1947;150:70–7.
[18] Hewetson JF, Rivera PV, Lemley PV, Pitt ML, Cresia DA, Thompson WL. A formalinized toxoid for protection of mice from inhaled ricin. Vaccine 1995;4:179–87.
[19] Ryan EJ, McNeela E, Pizza M, Rappuoli R, O’Neil L, Mills KH. Modulation of innate and acquired immune response by *Escherichia coli* heat-labile toxin, distinct pro- and anti-inflammatory effects of the nontoxic AB complex and the enzyme activity. J Immunol 2000;165:5750–9.
[20] Ryan EJ, McNeela E, Murphy GA, Stewart H, O’Hagan D, Pizza M, et al. Mutants of *Escherichia coli* heat-labile toxin act as effective mucosal adjuvant for nasal delivery of an acellular pertussis vaccine: differential effects of the nontoxic AB complex and enzyme activity on TH1 and Th2 cells. Infect Immun 1999;67:6270–80.
[21] Baudner BC, Balland O, Giuliani MM, Von Hoegen P, Rappuoli R, Betbeder D, et al. Enhancement of protective efficacy following intranasal Immunization with vaccine plus a nontoxic LTK63 mutant delivered with nanoparticles. Infect Immun 2002;70:4785–90.